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RESEARCH ARTICLE

Cis-vaccinic acid, Dibutyl-Cyanamide and 9, 12-Octadecadienoic acid from pistachio *(Pistachia vera)* seed coat (PSCE) induced nuclear damage and cytotoxicity in human colon cancer HT-115 cells

Mohammed Saeed Alkaltham*, Pandurangan Subash-Babu, Ahmad Mohammad Salamatullah, Ghalia Shamlan, Laila Al-Harbi, Ali A Alshatwi

Department of Food Science and Nutrition, College of Food and Agricultural Sciences, King Saud University, P. O. Box 2460, Riyadh 11451, Saudi Arabia

ABSTRACT

Current strategy for colorectal cancer (CRC) therapy like regular intake of non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of developing CRC but they induce the regression of adenomas. Even the CRC management technique include colectomy, have not yet been proven to reduce mortality. The present study aimed to reduce the susceptibility of CRC via controlling cellular oxidative stress, inflammation and caspase depended apoptosis in HT-115 human colorectal cancer cells using pistachio seed coat extract as nutritional therapy. Increasing concentrations of pistachio seed coat ethyl acetate extract (PSCE) were applied to HT-115 cells, which were incubated for 24 h and 48 h. The IC_{50} values were 14 mg/dl after 24 h, and and 7.5 mg/dl after 48 h. Acridine orange/ethidium bromide staining confirmed the presence of 32% early apoptotic, 27% pre-apoptotic, 12% apoptotic, and 6% necrotic cells after 48 h. PSCE at 14 mg/dl significantly increased the antioxidant capacity via the expression of CYP1A and GSK3β, and decreased inflammatory agents via decreasing NF-κb, TNF-α, COX-2 and PGE-2 expression three-fold after 48 h. The expression of the tumor suppression related genes p53 and mdm2, and the apoptosis related genes Bax, Bcl-2, Caspase 3, p21, and PCNA levels increased one-fold, and levels of mdm2, Bcl-2 and PCNA decreased after PME treatment of 48 h. PSCE effectively controlled colon cancer cell proliferation via the caspase-dependent mitochondrial mediated apoptotic pathway.

Keywords: *Pistachia vera*; cytotoxicity; Human colon cancer; Cis-Vaccininc acid; Apoptosis

INTRODUCTION

The pistachio (*Pistacia vera L.*), a member of family Anacardiaceae, the commercial production of which occurs mostly in Iran, the USA, Turkey and Syria (Özbek et al., 2018; Saitta et al., 2009). Pistachio is widely consumed, and is a good source of protein, fat, favorable fatty acids, vitamins, minerals and fiber. Pistachio also contains a crucial amount of phytochemicals, such as the carotenoids β-carotene and lutein, γ-tocopherol and phenolic compounds such as phenolic acids, flavonoids, lignans, anthocyanins and pro-anthocyanidins. These compounds provide the functionality to scavenge free radicals and reactive oxygen species (Madhavi and Salunkhe, 1995; Halvorsen et al., 2006; Bolling et al., 2011; Ozbek et al., 2018). Different species and different parts, including the fruit, leaves, resin and seed of pistachio, are rich sources of flavonoids, polyphenols and other phytochemicals which are well known for their biological properties, such as anti-inflammatory and antioxidant activities (Oskoueian et al., 2020; Goli et al., 2005; Alhariri et al., 2007; Sari et al., 2010; Hosseinzadeh et al., 2012; Abolhasani et al., 2018). Over the last decade, these natural components have received considerable attention because of their potential role in health and disease (Calixto et al., 2003; Zhou et al., 2009; Arcan and Yemenicioglu, 2009).

The phenolic compounds in pistachios have many potentially valuable biological properties, including anti-carcinogenic activities (Rajaei et al., 2010; Fathalizadeh

***Corresponding author:**
Dr. Mohammed Saeed AlkalthamDepartment of Food Science and Nutrition, College of Food and Agricultural Sciences, King Saud University, P. O. Box 246, 0, Riyadh 11451, Saudi Arabia. **Phone number:** +966114676839. **E-mail:** malkaltham@ksu.edu.sa

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et al., 2015; Glei et al., 2017). A study by Kocyigit et al. (2006) reported that the consumption of pistachio nuts decreased markers of oxidative stress in healthy subjects. A diet including pistachios has been shown to reduce inflammation and oxidative status in healthy young men (Sari et al., 2010). Seifaddinipour et al., (2020) found that pistachio hull extract possesses gallic acid and quercetin, which produces a caspase-dependent anticancer effect in breast cancer cells and animal models. Elyasi-Ghahfarokhi et al. (2021) reported a cytotoxic effect of laccase derived from *Pistacia atlantica* Desf. against HeLa and MDA-MB-231 cells. The beneficial health effects of plant-derived natural products are well known, due to their null or relatively lower side effects (Atanasov et al., 2015). However, studies exploring the underlying mechanisms of these beneficial properties of natural compounds are sparse (Unuofin and Lebelo, 2020).

Therefore, this study carried out to investigated the phytochemical composition of *Pistachio vera* seed coat ethyl acetate extract (PSCE). We determined its cytotoxic effects using measurements of oxidative stress, inflammatory markers and tumor suppressor gene expression in HT-115 human colon cancer cells. Pistachio seed coats are usually removed, and are not included in any food preparations, but the seed coat contains beneficial phytochemicals, as described above. The main aim was to evaluate the antioxidant, tumor suppressor and cytotoxic effects of pistachio seed coat extract, using HT-115 human colon cancer cells. There are currently no reports published on the cytotoxic, antioxidant, anti-inflammatory and pro-apoptotic properties of the seed coat of pistachio (*Pistachio vera*) in human colon cancer cells.

MATERIALS AND METHODS

Pistachio seed coat extract preparation

Fresh pistachios (*Pistacia vera* L.) with shells were purchased from the local hypermarket in Riyadh, Saudi Arabia. In order to remove the seed coat of the pistachios, the shells were removed from the seeds and immersed in 40°C water for five minutes. After completing the boiling step, the seed coats were steeped for four minutes, and then removed manually. The seed coats were left at room temperature to dry overnight. The dried seed coats were ground to powder using a commercial blender and then extracted with ethyl acetate using maceration method. Briefly, forty grams of seed coat powder was macerated with 200 ml of ethyl acetate with shaking at 50 rpm at room temperature for 48 h. The extract was collected and filtered through Whatman No.1 filter paper, and then a rotary evaporator at 45°C was used to evaporate the solvent. The residue obtained was stored in a refrigerator at −4°C in a sealed container under nitrogen gas until further use.

GC-MS analysis

The identification of the phytochemical components of the pistachios (*Pistacia vera* L.) seed coat ethyl acetate extract (PSCE) was performed using GC/MS (Alshatwi et al., 2018). GC-MS analyses were carried out using PerkinElmer Clarus 600 GC System fitted with an Rtx-5MS capillary column (30 m×0.25 mm inner diameter, 0.25 µm film thickness; maximum temperature 350°C), coupled to a Perkin Elmer Clarus 600C MS. For the detection step, the electron impact mode was selected with ionization energy of 70 eV, and electron multiplier voltage which obtained from auto tune. constant flow rate of 1.0 mL/min was performed using Ultra-high purity helium (99.99%). The temperature for the injection, transfer line and ion source were fixed at 290°C. A steadily increasing of oven temperature was planned to start from 60°C, held for 2 min, to 280°C, at a rate of 3°C/min. The dilution with ethanol (1/100, v/v) and filtration were completed before the extract injection. The diluted and filtrated extract volume of 1 µL injected with a split ratio of 30:1. The full-scan mass spectra collected for all data within the scan range 40–550 amu. The percentage of the peak area represent the percentage composition of the pistachio seed coat extract constituents (PerkinElmer Clarus, 600 GC System).

Cell lines

The HT-115 human colon carcinoma cell line was obtained from the National Center for Cell Science, Pune, India. The cells were incubated in a 5% CO_2 incubator at 37°C using RPMI-1640 medium containing 5% FBS, 100 U/ml penicillin and 100 µg/ml penicillin/streptomycin.

Cell viability assay

The *in vitro* cytotoxicity of PSCE was assayed using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Promega, Madison, WI) (Mosmann, 1983). HT-115 cells were treated with increasing concentrations of PSCE (0, 2.5, 5, 10, 20, or 40 mg/dl) in 96-well plates (Gibbco, USA) for 24 h and 48 h. After incubation for 24 h and 48 h, 20 µl of MTT solution was added to each well in the dark and incubated for 4 h at 37°C. Using micro plate reader at 492 nm, the absorbance of purple formazan crystals that produced by viable cells and dissolved in DMSO was measured.

Cell and nuclear morphology

HT-115 cells treated with 7.5 and 14 mg/dl doses of PSCE were analyzed for characteristic apoptotic and morphological changes using propidium iodide (PI) or acridine orange/ethidium bromide (AO/ErBr) staining after 48 h, to determine the effective doses (Leite et al.,

1999). Three hundred randomly selected stained cells were analyzed using an inverted fluorescence microscope and observed at 200X magnification. The number of cells showing pathological changes was calculated manually.

Quantitative polymerase chain reaction (qPCR) analysis

HT-115 cells treated with 7.5 and 14 mg/dl PSCE were used to synthesis cDNA using Fastlane® Cell cDNA kits (QIAGEN, Germany) after 48 h. The transcription of the oxidative stress related genes CYP1A, GSK3β, TNF-α and NF-κb, the pro-tumorigenic inflammatory genes COX-2 and PGE-2, and the apoptotic genes Bcl-2, Bax, Caspase 3, p53, mdm2, PCNA and p21 were quantified (Applied Biosystems, 7500 Fast, USA) using QIAGEN real-time SYBR Green/ ROX assay kits according to the manufacturer's instructions. Beta-actin was used as a reference gene. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative mRNA expression level of specific genes. In this method, ΔΔCt = (Ct, target gene of experimental group − Ct, β-actin of experimental group) − (Ct, target gene of control group − Ct, β-actin of control group) (Yuan et al., 2006).

Statistical analysis

SPSS version 26.5 software was used to analyze the statistical significance of the cytotoxicity and gene expression data. The values were analyzed using one-way analysis of variance followed by Tukey's test (Duncan, 1957). All results were presented as the mean ± SD of four replicates, and the differences were considered to be statistically significant at $p \leq 0.01$ and $p \leq 0.001$.

RESULTS

Gas chromatography-mass spectrometry analysis

The GC-MS chromatogram of PSCE is shown in Table 1. The 2011 version of the NIST/EPA/NIH Mass Spectral Database (NIST 11) library (https://www.nist.gov/system/files/documents/srd/NIST1a11Ver2-0Man.pdf) was used to compare and identify the phytochemicals based on the similarity of their spectral data and their pharmacological effects (Table 1). We found 99%-95% similarity with respect to the peak values and retention time. The polyphenols were primarily cis-vaccininc acid, dibutyl cyanamide and fatty acids, 9-octadecenoic acid, 9,12-octadecadienoic acid and hexadecenoic acid (spectral data not shown). Some of these polyphenols are known for their biological activity, whereas a few compounds still remain unexplored.

In vitro cytotoxic effect of PSCE

Cell viability was significantly decreased only at the highest dose of PSCE. Fifty percent of the HT-115 cell population was killed (IC_{50}) by PSCE at 7.5 mg/dl in 48 h, and by 14 mg/dl in 24 h. Therefore, 7.5 and 14 mg/dl of PSCE were selected as the optimum concentrations for subsequent cell and molecular biology investigations (Fig. 1).

Effects of PSCE on cell and nuclear morphology

Fig. 2 shows the propidium iodide (PI) staining of PSCE treated cells. Using this staining we observed characteristic irregular and horseshoe-shaped nuclei. This observation indicated the presence of endoplasmic reticulum stressed cells and apoptotic cells (Fig. 2). There were no noticeable changes in control cells, even when they were clearly

Table 1: Composition analysis using GC-MS for pistachio seed coat ethyl acetate extract (PSCE) shown 95%–99% similarity in the database for fatty acids

S. No	Compound Name	Peak area (%)
1	Carbamic acid, ethyl-, methyl ester	0.25
2	1,2,3-Propanetriol, monoacetate	0.86
3	1,2,3-Propanetriol, monoacetate	0.28
4	1,2,3-Propanetriol, diacetate	0.28
5	1,2,3-Propanetriol, monoacetate	0.48
6	1,2,3-Propanetriol, monoacetate	0.29
7	2-ethyl-5-methyl-Pyrazine,	0.34
8	Dibutyl- Cyanamide.	0.20
9	Methyle.beta. -d-galactopyranoside	1.22
10	4,6-Di-O-methyl-. alpha. -d-galactose	0.52
11	n-Hexadecanoic acid	6.20
12	9,12 -Octadecadienoic acid (Z-Z)-	0.18
13	9,12 -Octadecadienoic acid (Z-Z)-	0.91
14	9-Octadecenoic acid, methyl ester	1.03
15	9,12 -Octadecadienoic acid (Z-Z)-	12.80
16	6-Octadecenoic acid (Z-Z)-	9.07
17	Cis-Vaccenic acid	1.82
18	9,12-Octadecadienoic acid (Z-Z)-	0.95
19	2-hydroxy-Cyclopentadecanone	0.10
20	Hexadecanoic acid, 2-hydroxy-1 - (hydroxymethl) ethyl ester	0.27
21	Octadecanoic acid, 2-hdroxy-1- (hydroxymethyl) ethyl ester Cyclononanone	1.12

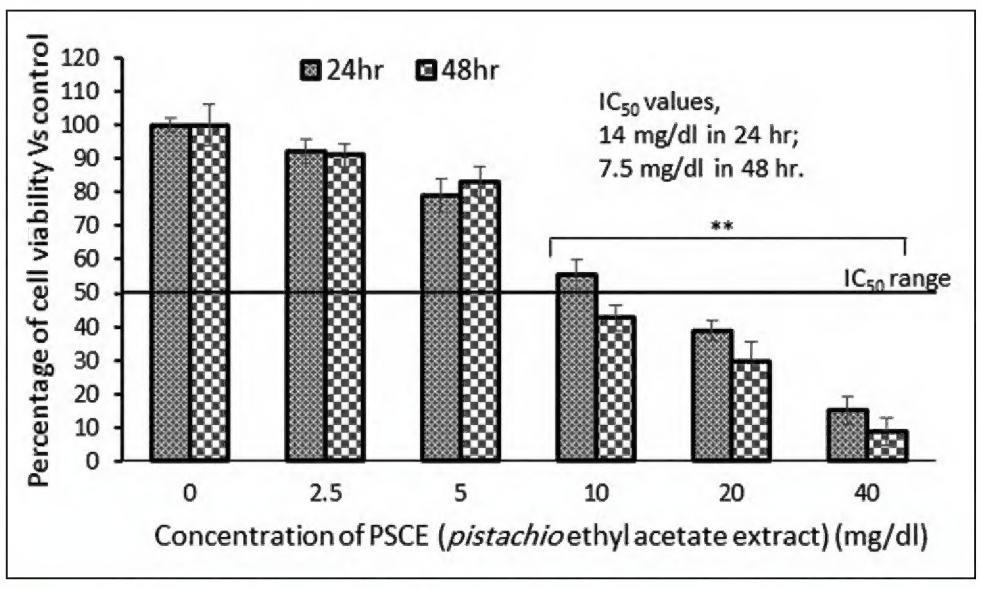

Fig 1. *In vitro* cytotoxic effect of pistachio seed coat ethyl acetate extract (PSCE) on HT-115 cells after 24 h and 48 h. Data are expressed as the mean ± SD (n = 6). * $p \leq 0.05$ & ** $p \leq 0.001$ vs. untreated control.

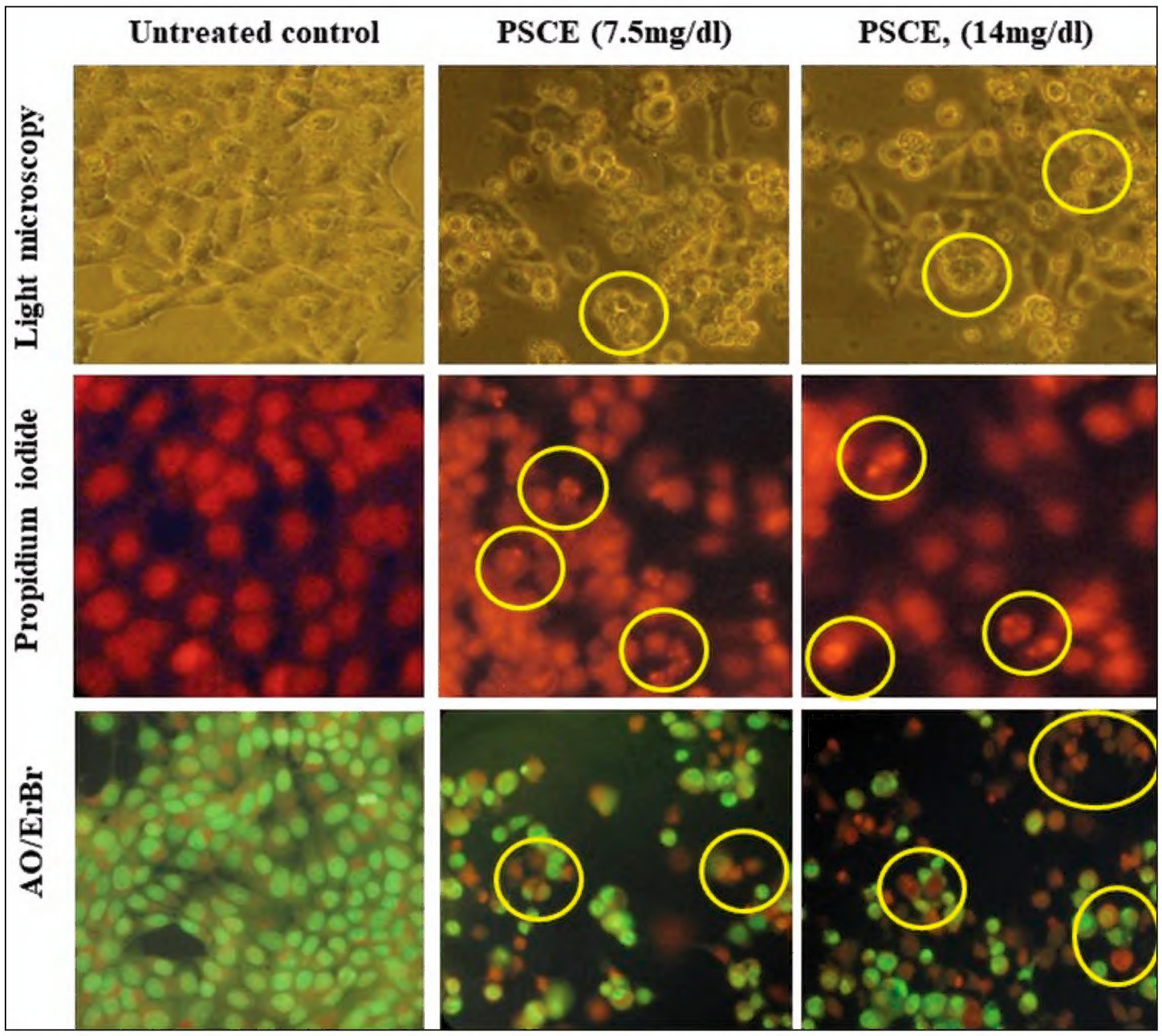

Fig 2. Light microscopy, propidium iodide and AO/ErBr-stained cells of pistachio seed coat ethyl acetate extract (PSCE) treated human colon cancer HT-115 cells after 48 h (200 x). *In fig. 2, PI staining of 7.5mg and 14mg of PSCE treated HT-115 cells; showing abnormal nuclei, horseshoe-shaped nucleus and cell death was evidenced by the florescence morphology. *In fig. 2, AO/ErBr staining of 7.5mg and 14mg of PSCE treated HT-115 cells showing proapoptotic-bright green, early apoptotic -light green, late apoptotic- orange and necrotic cells- bright red color were highlighted by circle.

observed under light microscopy (Fig. 2). Using AO/ErBr staining, the 14 mg/dl dose of Pistachio ethyl acetate extract showed the presence of pre-apoptotic cells (dark green), early apoptotic cells (light green), late apoptotic cells (orange) and necrotic cells (red) (Fig. 2) compared with control. Manual counting under a florescence microscopic indicated the presence of 32 % early apoptotic, 27 % pre-apoptotic, 12 % late apoptotic, and 6% necrotic cells after a 14 mg/dl dose of PSCE in HT-115 cells (Fig. 3).

Effect of PSCE on oxidative stress and pro-tumorigenic inflammatory gene expression

We analyzed the oxidative stress and inflammatory genes CYP1A, GSK3β, TNF-α, NF-κB, COX-2 and PGE-2 after 48 h of PSCE treatment of HT-115 cells (Fig. 4a). We found an increased expression of COX-2 and PGE-2 levels in untreated HT-115 cells. Treatment with 14 mg/dl of PSCE decreased the COX-2 and PGE-2 expression levels by three-fold after 48 h. A single-fold decrease of NF-κB and TNF-α expression was also observed, and CYP1A and GSK3β expression levels were increased by a single fold in 48 h when compared to untreated control.

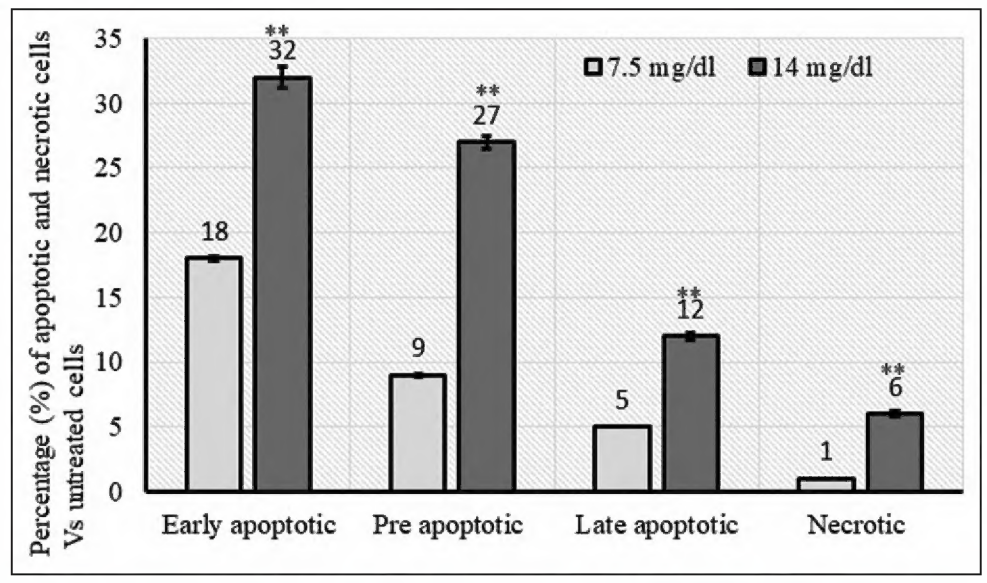

Fig 3. The percentage of early, pre, late apoptotic and necrotic cells of pistachio seed coat ethyl acetate extract (PSCE) treated human colon cancer HT-115 cells after 48 h. Data presented as the means ± SD are shown; n = 6; values sharing a common superscript as * $p \leq 0.05$ & ** $p \leq 0.001$ compared with untreated control.

Effect of PSCE on mitochondria-dependent apoptotic gene expression

Apoptosis related gene expression levels in PSCE treated HT-115 cells after 48 h are shown in Fig. 4b. Tumor suppressor related p53 and mdm2 mRNA expression was significantly increased in PSCE treated HT-115 cells

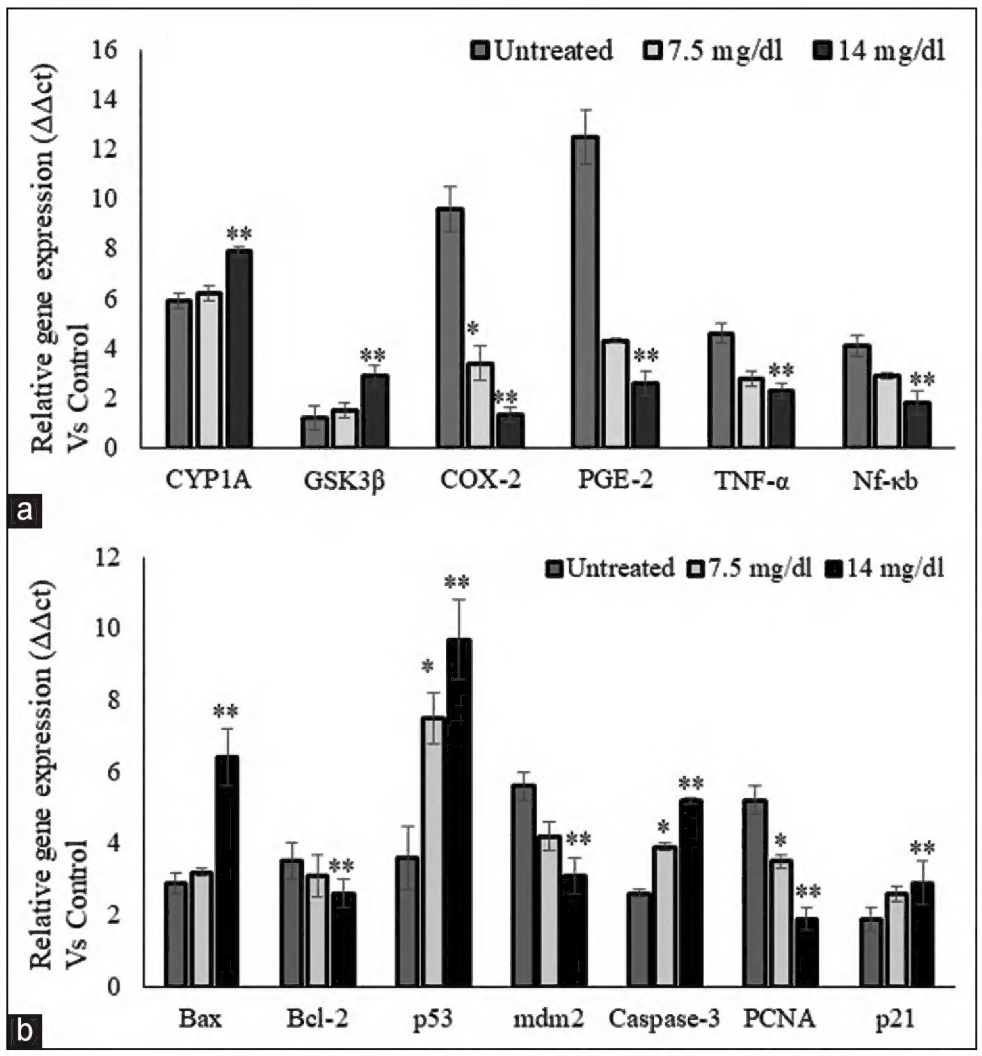

Fig 4. The dose responsive effect of pistachio seed coat ethyl acetate extract (PSCE) treated HT-115 cells on immunoregulatory, tumor suppression and apoptosis related gene expression levels after 48 h. Data presented as the means ± SD are shown; n = 6; values sharing a common superscript as * $p \leq 0.05$ & ** $p \leq 0.001$ compared with untreated control.

compared to untreated cells. The levels of mitochondria-dependent apoptotic stimulator mRNAs such as Bax, Bcl-2, Caspase 3, p21 and PCNA were increased two-fold, and Bcl-2 and PCNA expression was significantly decreased after 48 h in PSCE treated HT-115 cells. In all of the parameters, a dose of 14 mg/dl produced significantly higher expressions of pro-tumorigenic inflammatory and mitochondria-dependent apoptotic gene expression than the lower dose of 7.5 mg/dl of PSCE.

DISCUSSION

GC-MS characterization of PSCE confirmed the presence of polyphenols, cis-vaccinic acid, dibutyl cyanamide and the fatty acids hexadecenoic acid, 9,12-octadecadienoic acid and 9-octadecenoic acid. Previously, cis-vaccinic acid and 9,12 octadecanoic acids have been reported as having beneficial cytotoxicity and anticancer effects *in vitro* and *vivo* (Zahouani et al., 2020). A significant dose dependent effect on the cell viability was observed in HT-115 cells treated with concentrations of PSCE ranging from 2, 5, 10, 20 and 40 mg/dl, with the highest dose exerting the maximum cytotoxic effects. Fifty percent of cell death was observed at 7.5 mg/dl concentration of extract after 48 h of treatment, and with 14 mg/dl concentration after 24 h of treatment, indicating the efficacy and potential of the extract in the inhibition of cell growth, even at lower doses. The cytotoxic effects of the extract were consistent with previous studies, in which different species of pistachio are reported to inhibit the growth of different cell types (Ben Ahmed et al., 2021, Seifaddinipour et al., 2020, Sarkhail et al., 2019).

These findings were validates by the PI staining results of control and PSCE treated HT-115 cells. The PI stained nuclei of untreated cells showed stressed endoplasmic reticulum and irregular and horseshoe-shaped cells compared with the normal nuclei of cells treated with PSCE. Changes in cell morphology were visualized using AO/ErBr staining of cells. Of the cells, 27 % were pre-apoptotic, 32 % were early apoptotic, and 12 % were late apoptotic. In the PSCE-exposed cells, 6% were necrotic. Overall, the manual counting of cells revealed 71 % of cells to be apoptotic and 6% to be necrotic after treatment with 14 mg/dl of pistachio extract, while no apoptotic or necrotic cells were observed in control cells. Based on these data, it appears that pistachio extract induces apoptosis, but not necrosis, in cells (Fathalizadeh et al., 2015). Since cell and nucleus morphology is indicative of apoptosis, we further investigated the gene expression of pro-tumorigenic and anti-apoptotic molecules.

The cells treated with 14 mg/dl of PSCE were found to contain significantly elevated levels of CYP1A and GSK3β compared to those in the control. CYP1A and GSK3β play a major role in cellular defense against oxidative stress, and their levels are downregulated in cancer cells (Subash-Babu et al., 2017). COX-2 and PGE-2 are known promoters of carcinogenesis, and their levels have been reported to be augmented in cancer cells (Wang and Zhang, 2018). The levels of COX-2 and PGE 2 were highly upregulated in control cells, but their levels dramatically declined after treatment with PSCE. Thus, increased levels of CYP1A and GSK3β. and decreased COX-2 and PGE2 levels in PSCE treated cells compared to control indicate the antioxidant capacity of PSCE. TNF-α is an inflammatory cytokine produced by macrophages and monocytes, and participates in several signaling pathways (Lagha and Grenier, 2019). NF-κβ is a major transcription factor activating pro-inflammatory and tumorigenic genes (Guolan et al., 2018). In the present study, both of these pro-inflammatory molecules were elevated in HT-115 cells. Treatment of cells with *Pistachio vera* extract led to significant decreases in TNF-α and NF-κβ levels, underscoring the anti-inflammatory potential. These data are in accordance with previous studies, in which different species of pistachio have been demonstrated to exert antioxidant and anti-inflammatory effects under different experimental settings (Zahouani et al., 2020, Asbaghi et al., 2020, Ostovan et al., 2020, Abidi et al., 2017).

Real-time PCR data revealed a significant increase in the expression of the pro-apoptotic proteins Bax, p53, Caspase-3 and p21, and a significant decrease in the expression of anti-apoptotic proteins including Bcl-2, mdm2 and PCNA in cells treated with PSCE compared to those in control cells. These findings are indicative of the pro-apoptotic nature of pistachio seed coat extract, and are in line with previous studies, which have demonstrated pro-apoptotic properties of various species of pistachio under different experimental conditions (Koyuncu et al., 2020, Seifaddinipour et al., 2020, Jaafari-Ashkvandi et al., 2019, Seifaddinipour et al., 2018).

CONCLUSIONS

These data demonstrate the anti-inflammatory and antioxidant properties of the ethyl acetate extract of the pistachio seed coat in HT-115 cells. Pistachio extract produced a cytotoxic effect on HT115 cells, and induced cell and nuclear damage, the expression of pro-apoptotic genes, and attenuation of anti-apoptotic gene expression. These effects appear to be mediated by the ability of the extract to reduce oxidative stress and inflammation, and induce apoptosis in HT115 colon cancer cells. Thus, regular consumption of soaked pistachios with seed coat may have beneficial health effects.

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

All the authors are declaring that they have no conflicts of interest.

Authors' contributions

Conceptualization: Mohammed Alkaltham and P. Subash-Babu; writing—original draft preparation: Mohammed Alkaltham, P. Subash-Babu; writing—review and editing: Ghalia Shamlan; funding acquisition: Ali Alshatwi, Ahmad Mohammad Salamatullah; supervision: P. Subash-Babu. All authors have read and agreed to the published version of the manuscript.

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